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# Natural Resistance of Human Immunodeficiency Virus Type 1 to the CD4bs Antibody b12 Conferred by a Glycan and an Arginine Residue Close to the CD4 Binding Loop<sup>▽</sup>

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**The human monoclonal antibody b12 recognizes a conserved epitope on gp120 that overlaps the CD4 binding site. b12 has neutralizing activity against diverse human immunodeficiency virus type 1 (HIV-1) strains. However, we recently reported that b12 sensitivity of HIV-1 envelopes amplified from patient tissues without culture varied considerably. For two subjects, there was clear modulation of b12 sensitivity, with lymph node-derived envelopes being essentially resistant while those from brain tissue were sensitive. Here, we have mapped envelope determinants of b12 resistance by constructing chimeric envelopes from resistant and sensitive envelopes derived from lymph node and brain tissue, respectively. Residues on the N-terminal flank of the CD4 binding loop conferred partial resistance. However, a potential glycosylation site at residue N386 completely modulated b12 resistance but required the presence of an arginine at residue 373. Moreover, the introduction of R373 into b12-sensitive NL4.3 and AD8 envelopes, which carry N386, also conferred b12 resistance. Molecular modeling suggests that R373 and the glycan at N386 may combine to sterically exclude the benzene ring of b12 W100 from entering a proximal pocket. In summary, we identify residues on either side of the CD4 binding loop that contribute to b12 resistance in immune tissue *in vivo*. Our data have relevance for the design of vaccines that aim to elicit neutralizing antibodies.**

The human immunodeficiency virus type 1 (HIV-1) envelope on virions is made of a surface (SU) gp120 and transmembrane (TM) gp41 that are assembled as trimeric glycoprotein spikes. Binding of CD4 to the trimeric spike induces conformational changes in gp120 that result in the formation of a binding site for the coreceptor. The binding of gp120 to coreceptors then triggers further conformational changes that release the fusion domain of gp41 and initiate fusion between virion and host cell membranes (2). gp120 was first crystallized in a complex with the two N-terminal domains of CD4 and a human gp120-specific monoclonal antibody (MAb) (17b) (7). The gp120 construct used to obtain this structure was deleted for variable loops V1 to V3 and for several potential glycosylation sites (PGSSs). Nevertheless, the structure showed that the gp120 core consists of an inner domain of gp120 (which would include the N and C termini of gp120 and sites predicted to interact with gp41) connected to an outer domain via a “bridging sheet” composed of four  $\beta$ -strands. CD4 binds to a site that comprises several sites on different gp120 regions that are brought together on binding into a depression between the three gp120 domains.

Previously, we reported that CCR5-using, R5 envelopes varied considerably in macrophage tropism (11, 13). For example, highly macrophage-tropic R5 envelopes were prevalent in brain tissue of patients with neurological complications includ-

ing dementia but were less frequent in lymph node (LN), blood, and semen (11, 13). These envelopes were characterized by their capacity to infect cells via low levels of CD4 (11, 13). In addition, Dunfee et al. (4) described an envelope polymorphism at residue 283 in the C2 part of the CD4 binding site. Thus, N283 was associated with 41% of envelopes present in the brain of subjects with HIV-associated dementia and with only 8% in non-HIV-associated dementia subjects (4). We also noted that N283 was present in over 50% of highly macrophage-tropic envelopes from brain but infrequent in envelopes from LN, blood, and semen (13). N283 may form a hydrogen bond with Q40 on CD4 (more readily than the usual T283 residue) and confer a higher gp120:CD4 affinity (4). Nonetheless, not all macrophage-tropic R5 envelopes carry N283, and additional unknown determinants must also exist.

More recently, we have shown that most macrophage-tropic brain envelopes tested were sensitive to the CD4 binding site MAb b12, while the majority of non-macrophage-tropic R5 envelopes from LN were resistant. For example, for two subjects, several envelopes amplified from LN tissue were resistant to b12, while those from brain were sensitive, thus revealing clear inpatient and tissue-specific variation in b12 sensitivity. These results suggested to us that HIV-1 replication in the brain may result in the evolution of envelopes that carry a more exposed CD4bs which would contribute to an increased affinity for CD4 but increase the vulnerability of envelopes to CD4bs Abs. Moreover, the blood-brain barrier excludes most immunoglobulin from the brain and may thus provide an ideal environment for the evolution of such variants.

Several studies show that b12 carries the potential to neutralize diverse HIV-1 isolates (9, 10), while the CD4bs and the

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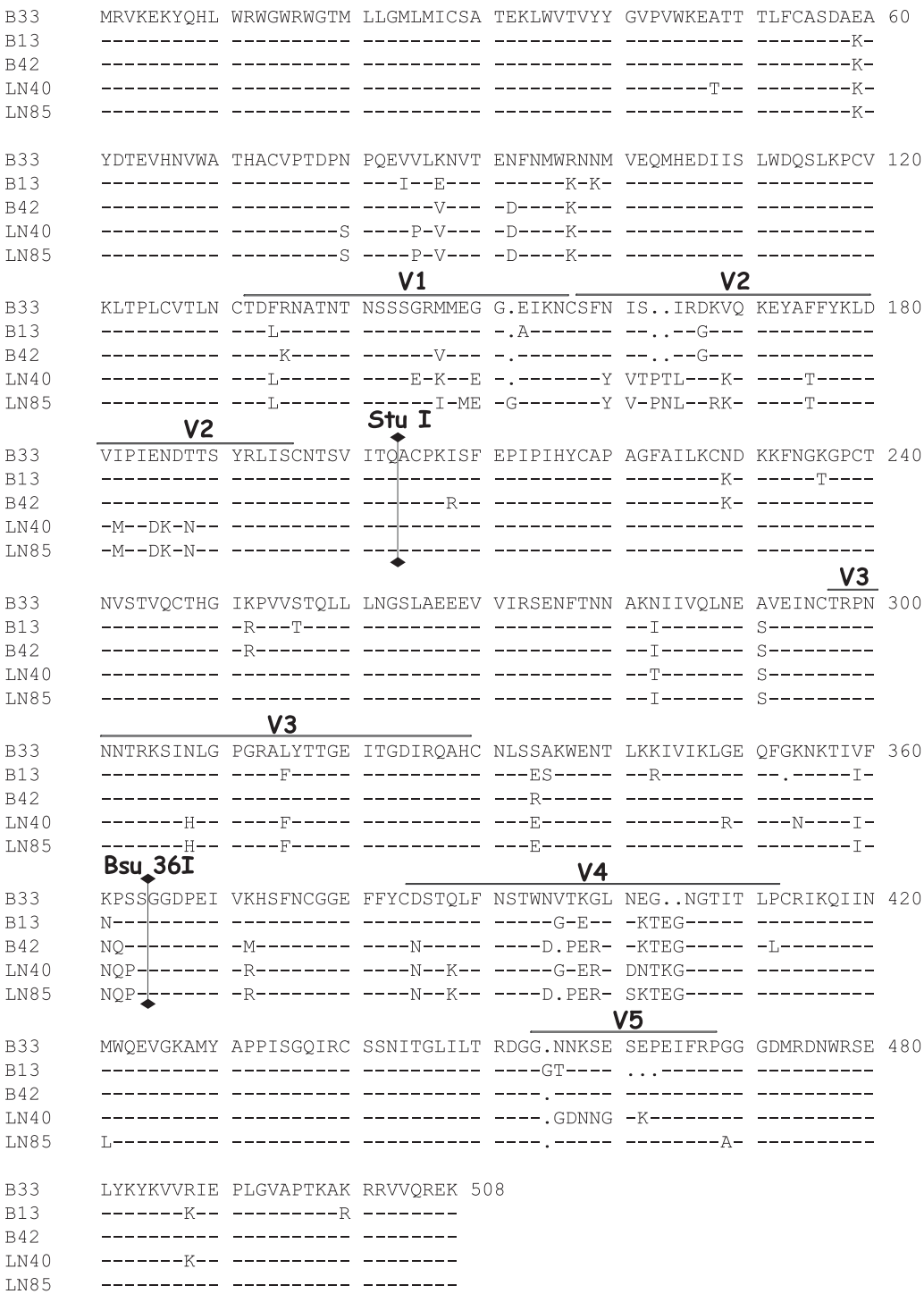


FIG. 1. gp160 amino acid sequence alignment for NA420 envelopes. StuI and Bsu36I restriction sites used to prepare chimeric envelopes occur after residues Q203 and S364, respectively. Note that residue numbering for envelopes in this figure does not precisely follow HXBc2 numbering, which is used throughout the text.

b12 epitope are under intense investigation as potential candidates for vaccines. It is therefore important to understand how variation in b12 sensitivity arises in vivo and to elucidate the mechanisms of resistance involved. It will also be critical to understand how envelope mutations that confer b12 resistance affect exposure of the b12 epitope and the CD4bs. Here, we

have mapped envelope determinants involved in b12 resistance by constructing chimeric envelopes from a b12-sensitive brain envelope and a resistant LN envelope from the same patient. We show that determinants on the N-terminal flank of the CD4 binding loop conferred partial resistance to b12. Moreover, a glycosylation site at N386 conferred complete b12 re-

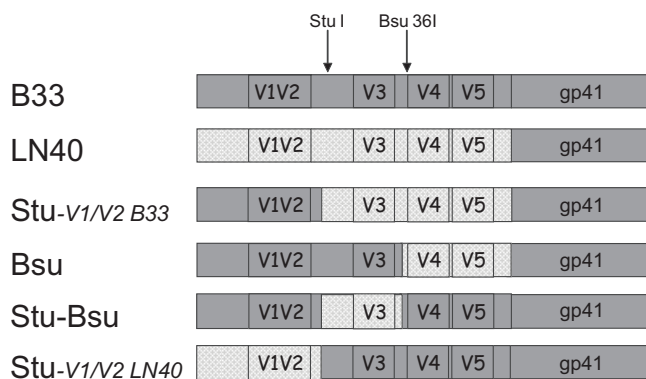


FIG. 2. Chimeric B33/LN40 envelopes constructed for mapping determinants of b12 sensitivity.

sistance but required the presence of an arginine at residue 373. R373 and N386 are close to the CD4 binding loop and together appear to sterically prevent the benzene ring on the side chain of W100 of b12 from penetrating a pocket close to the two gp120 residues.

#### MATERIALS AND METHODS

**Cells.** 293T cells (3) were used to prepare env<sup>+</sup> pseudovirions by transfection. HeLa TZM-BL cells were used to titrate env<sup>+</sup> pseudovirions and to evaluate HIV-1 neutralization. HeLa TZM-BL cells express high levels of CD4 and CCR5 and contain  $\beta$ -galactosidase and luciferase reporter genes under the control of an HIV long terminal repeat (14).

**Patient tissue samples and molecular constructs of pseudovirions.** HIV envelopes used here were described previously (11, 13). Envelopes B33, B42, and LN40 were derived from subject NA420, a heterosexual patient who died of AIDS with no cognitive impairment and sparse giant-cell encephalitis. Samples from temporal lobe brain and LN tissue obtained at autopsy were frozen at  $-80^{\circ}\text{C}$ , and DNA was extracted as described previously (15). PCR amplification of complete envelopes from tissue DNA was performed as described previously (11). NA420 envelope genes were cloned into conserved KpnI restriction sites into pSVIIIenv (6) and into pBluescript for direct mutagenesis.

**Mutagenesis.** Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene Inc.) using gp160<sup>+</sup> pBluescript plasmids as templates and mutagenic primers to introduce the desired mutations. The presence of each mutation was confirmed by sequencing. Mutated gp160s were cloned into pSVIIIenv via conserved KpnI sites.

**Production and titration of env<sup>+</sup> pseudovirions.** The env<sup>+</sup> pNL4.3 construct and pSVIIIenv expression vectors were described previously (11, 13). Pseudovirions carrying patient envelopes were produced by cotransfection of env<sup>+</sup> pSVIIIenv with env<sup>+</sup> pNL4.3 into 293T cells using calcium phosphate. Cell supernatants carrying pseudovirions were harvested 48 h after transfection, clarified ( $1,000 \times g$  for 10 min), aliquoted, and stored at  $-152^{\circ}\text{C}$ .

Pseudovirions carrying patient-derived envelopes were titrated on HeLa TZM-BL cells using the long terminal repeat-controlled  $\beta$ -galactosidase reporter gene to identify infected cells as described previously (16). Since env<sup>+</sup> pseudovirions undergo only a single round of replication, focus-forming units/ml were estimated by counting individual or small groups of blue-stained cells.

**Neutralization and inhibition assays.** Neutralization was measured as a reduction in  $\beta$ -galactosidase reporter gene expression after infection of HeLa TZM-BL cells with MAb-treated pseudovirions. Two hundred microliters of HeLa TZM-BL ( $4 \times 10^4$  cells/ml) was added to each well in 96-well luminescence plates (Corning Inc.) 1 day before neutralization assays. Two hundred focus-forming units of pseudovirions was incubated with twofold dilutions of b12 for 2 h at  $37^{\circ}\text{C}$ . Fifty microliters of this mixture was incubated with HeLa TZM-BL cells at  $37^{\circ}\text{C}$  overnight before being removed, and 200  $\mu\text{l}$  of growth medium was added. Seventy-two hours after incubation, the luminescence reaction was initiated by adding 100  $\mu\text{l}$  of Beta-Glo (Promega Inc.) with 100  $\mu\text{l}$  of Dulbecco modified Eagle medium (no phenol red) per well. Luminescence was measured 30 min later. Percent neutralization was defined by the formula  $(1 - p/n) \times 100$ , where  $p$  is the mean luminescence value produced in the presence of

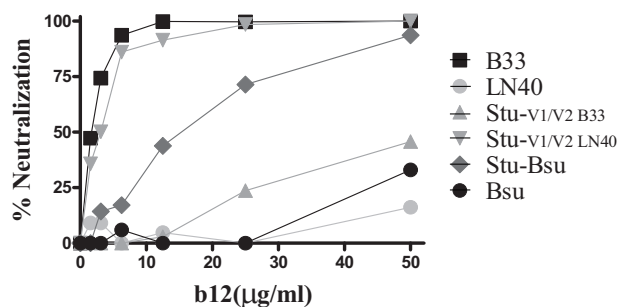


FIG. 3. Identification of gp120 regions responsible for b12 resistance by using chimeric constructs made from B33 and LN40 envelopes.

b12 MAb and  $n$  is the mean luminescence value produced in the absence of b12 MAb (positive control).

**ELISA for soluble gp120.** Costar 96-well enzyme-linked immunosorbent assay (ELISA) plates (catalog no. 3690; Corning Inc.) were coated with 250 ng/well of sheep anti-gp120 Ab D7324 (catalog no. 6205; Clinique, Inc.) in phosphate-buffered saline (PBS). After overnight incubation at  $4^{\circ}\text{C}$ , plates were washed twice with PBS-0.05% Tween, incubated for 1 h at room temperature (RT) with blocking solution (3% bovine serum albumin in PBS), and washed four times with PBS-0.05% Tween. Fifty microliters/well of env<sup>+</sup> pseudovirion dilutions (in 1% bovine serum albumin-PBS-0.02% Tween) was added for 2 h at RT. After 10 washes, appropriate dilutions of b6 and b12 MAbs or QCMix were incubated at RT for 2 h and washed 10 times. Fifty microliters/well of goat anti-human immunoglobulin G, F(ab')<sub>2</sub> fragment-specific, calf intestinal alkaline phosphatase conjugate (Pierce 31312) diluted 1:1,000 in dilution buffer was added to plates. After 1 h of incubation at  $37^{\circ}\text{C}$ , an AMPAK kit (Argene Inc.; K6200) was used to detect alkaline phosphatase activity, following kit instructions. Plates were read at 492 nm after stop solution was added.

**PyMol analyses.** The structure of HIV-1 gp120 complexed with the broadly neutralizing CD4 binding site Ab b12 was described by Zhou et al. (17). The 2NY7.pdb file of the gp120:b12 structure was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank web page.

#### RESULTS

**Envelopes derived from the LN and brain of patient NA420 vary in biological properties.** Previously we described five R5 envelopes that were amplified from brain and LN tissue of a heterosexual HIV-1<sup>+</sup> patient (NA420) who died of AIDS with neurological complications (11, 13). The brain-derived R5 envelopes were macrophage tropic, and we have shown recently that they are sensitive to neutralization mediated by the human MAb b12 (12). In contrast, the LN-derived envelopes infected macrophages very inefficiently and were resistant to b12. Figure 1 shows the amino acid alignment for gp120 of these five envelopes.

**Identification of gp120 regions involved in b12 sensitivity and resistance.** Chimeric envelopes were constructed from B33 and LN40 envelopes in pSVIIIenv (Fig. 2). The LN40 parental construct used here already carried the gp41 region of B33, greatly simplifying the design of chimeric envelopes. This LN40 construct had previously been shown to be resistant to b12-mediated neutralization, while B33 was sensitive.

The sensitivity of the chimeric envelopes to b12 neutralization was tested using a single-round pseudovirion infectivity system (see Materials and Methods). The Stu-V1/V2 LN40 and Stu-V1/V2 B33 chimeras exchange a region that includes the V1V2 loops. These chimeric envelopes conferred b12 sensitivity similar to that conferred by the parental envelopes (Fig. 3),

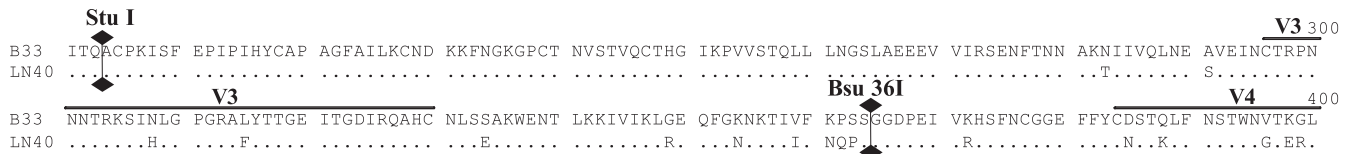


FIG. 4. Amino acid sequence alignment for NA420 envelopes. Amino acids from StuI to the V4 loop are shown. This region covers the residues identified here as determinants in b12 resistance. StuI and Bsu36I restriction sites occur after residues Q203 and S364, respectively. Note that residue numbering for envelopes in this figure does not precisely follow HXBc2 numbering, which is used throughout the text.

indicating that the V1V2 loops had little effect on b12 sensitivity. The Stu-V1/V2 B33 chimera also shows that determinants that confer resistance to b12 lie in a region of gp120 that includes the V3, V4, and V5 loops. The Stu-Bsu and Bsu chimeras split this region into two. The Stu-Bsu chimera is comprised of B33 which carries a region of LN40 gp120 with the V3 loop and sequences flanking the N-terminal side of the CD4 binding loop (1). In the background of B33, this region conferred partial resistance to b12, conferring a shift in 50% infective dose from 1.7 to 13.86  $\mu\text{g/ml}$ . In contrast, a region of LN40 comprising the conserved SGGDPE part of the CD4 binding loop and the V4 and V5 loops in a background of B33 (Bsu chimera) conferred optimal resistance to b12.

**The role of the Stu-Bsu region of gp120 in b12 resistance.** We next examined the role of the StuI-Bsu36I region of gp120 in conferring partial b12 resistance. This region includes the V3 loop and the N-terminal flank of the CD4 binding loop and contains 11 amino acid differences between B33 and LN40 including an N283T change for LN40 in the C2 CD4 binding site (Fig. 4). The presence of asparagine at residue 283 was reported to predominate in HIV-1 envelope sequences in brain tissue of patients with dementia and was associated with an enhanced macrophage tropism (4). We also reported that N283 was predominant in envelopes amplified from brain tissue but infrequent in envelopes from LN, blood, and semen (13). We tested whether the presence of asparagine, threonine, or isoleucine (the most common residues) at this position (283) had any effect on b12 sensitivity. B33 mutants carrying N, T, or I at residue 283 were all sensitive to b12 neutralization, while LN40 mutants carrying N, T, or I at residue 283 were all resistant (data not shown). Thus, residue 283 does not affect b12 sensitivity for the envelopes tested here.

Other amino acid differences between B33 and LN40 in this region include an alanine-to-serine switch (residue 291) which confers the presence of a PGS in LN40 and two differences in the V3 loop (N308H and L315F) (Fig. 4). There are also five differences (present in both LN40 and LN85) between the end of the V3 loop and the Bsu36I restriction site including the substitution of four of five consecutive residues (VFKPS $\Rightarrow$ IFNQP) flanking the GDPE motif in the CD4 binding loop. We constructed mutant versions of B33 carrying various combinations of the amino acids present in both b12-resistant LN40 and LN85 but absent in b12-sensitive B33. We first tested B33 mutants that carried SHFE, INQP, or SHFEINQP substitutions (Fig. 4). B33 (SFHE) was only marginally less sensitive to b12 than was B33 (Fig. 5A). However, B33 (INQP) showed a marked shift toward b12 resistance, yet it was still more sensitive than the Stu-Bsu chimeric envelope that carries the entire Stu-Bsu region from LN40. B33 (SFHEINQP) combines

eight implicated substitutions and conferred resistance to b12 similar to that conferred by the partially resistant Stu-Bsu chimeric env. We next investigated the role of residues in the upstream flank of the CD4 binding loop. B33 mutants carrying Q, P, QP, NQP, or INQP (residues present in LN40) were partially resistant to b12, with the QP motif consistently conferring b12 resistance closest to that conferred by the Stu-Bsu

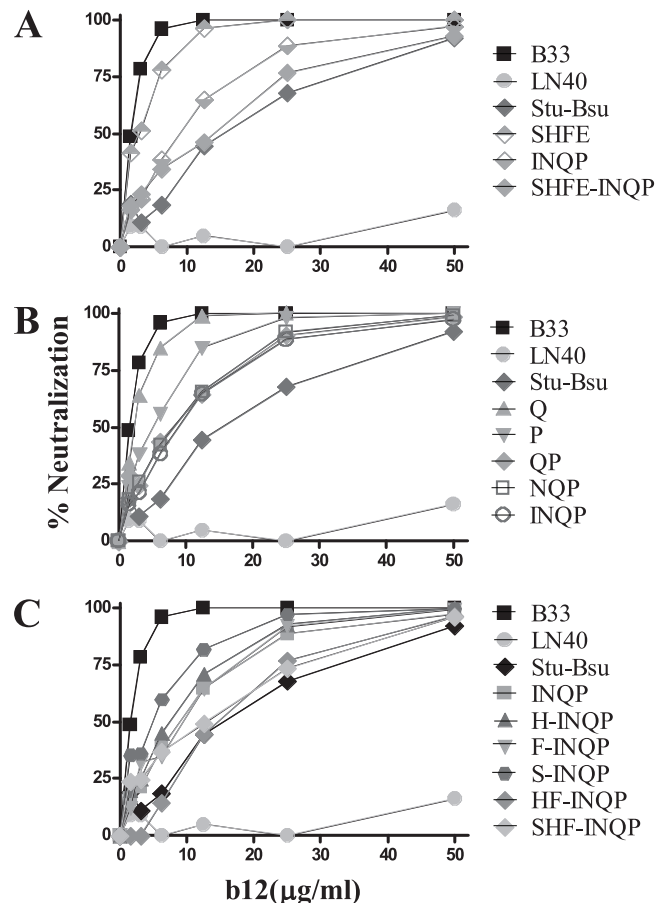


FIG. 5. Amino acids flanking the CD4 binding loop influence b12 sensitivity. Residues present in the LN40 Stu-Bsu fragment but absent in B33 were introduced into B33. (A) B33 substitutions were made for residues on the N-terminal flank of the CD4 binding loop (INQP [Fig. 4]) and for residues upstream (SHFE) or both. Residues on the CD4 binding loop flank confer partial resistance, and this is enhanced by upstream residues. (B) Residues QP on the CD4 binding loop flank confer the most resistance. (C) Residues H and F in the V3 loop combine with CD4 binding loop flanking residues to confer the partial b12 resistance shown by the Stu-Bsu LN40 fragment.



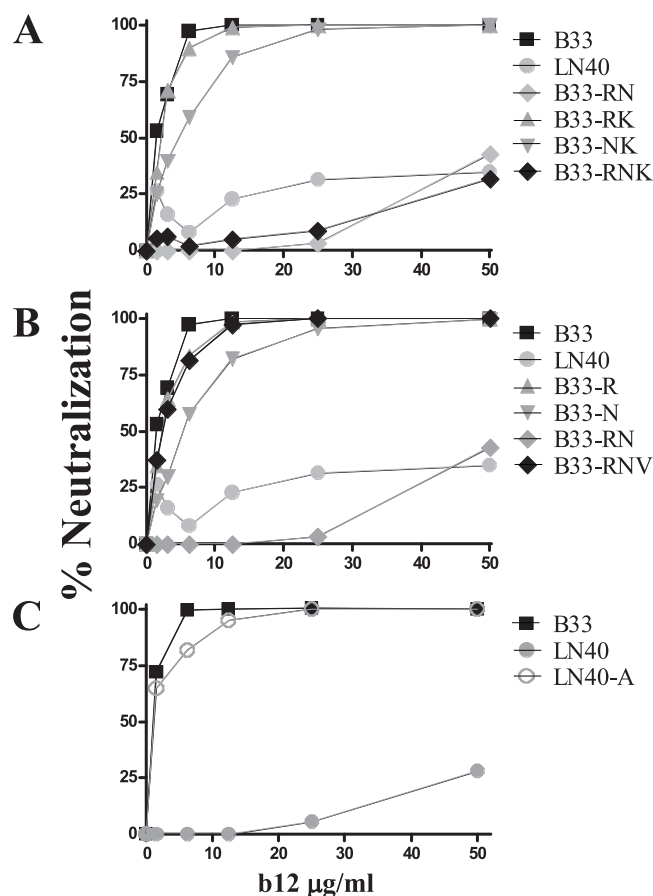


FIG. 6. Resistance to b12 is conferred by a PGS at residue 386 but is modulated by the residue at 373. (A) Double and triple B33 mutants carrying substitutions at 373, 386, or 389. The combination of R373 and N386 is required for optimal resistance to b12. (B) Neither R373 nor N386 alone confers b12 resistance. However, b12 resistance conferred by R373/N386 is abrogated if the PGS at N386 is eliminated by a T388V substitution. Thus, R373 and the glycan at N386 are required for maximal b12 resistance. (C) T388A in LN40 retains the asparagine at 386 but eliminates the glycosylation signal. T388A confers sensitivity to b12 for LN40.

chimera (Fig. 5B). Finally, we analyzed B33 carrying INQP and various combinations of the SFHE substitutions. B33 mutants that carried V3 loop residues H and F in combination with INQP conferred resistance to b12 similar to that conferred by the Stu-Bsu chimera (Fig. 5C). In summary, QP substitutions located in the upstream flank of the CD4 binding loop confer partial resistance to b12. However, this resistance is enhanced by upstream residues, 308H and 317F, within the V3 loop.

**A PGS (N386) in the C-terminal region of gp120 confers optimal b12 resistance but depends on R373.** The sensitivity of chimeric B33/LN40 envelopes to b12 implicated a region from the Bsu36I restriction site to the gp120/gp41 junction for optimal b12 resistance. In this region, there are several differences in the V4 and V5 loops between B33 and LN40. However, we focused on differences closest to the CD4 binding loop (Fig. 4) including K/R, D/N, and Q/K substitutions at residues 373, 386, and 389, respectively. The D386N change results in the loss of a PGS in B33. The PGS at 386 was recently implicated in b12 sensitivity and macrophage tropism (5).

B33 mutants carrying double (K373R/D386N, D386N/Q389K, or K373R/Q389K) or triple (K373R/D386N/Q389K) substitutions were tested for b12 sensitivity. K373R/Q389K had no effect on b12 sensitivity (Fig. 6A). However, while D386N/Q389K conferred a small shift toward resistance, K373R/D386N and K373R/D386N/Q389K conferred complete resistance. These observations implicate the PGS at residue 386; however, optimal b12 resistance was observed only when N386 was present together with R373.

We next tested B33 mutants K373R and D386N as well as a triple mutant which contained K373R/D386N (which confer b12 resistance) but also T388V to abrogate the PGS at N386 (Fig. 6B). K373R alone had no effect on b12 sensitivity. The D386N substitution introduces the PGS at residue 386 and conferred a modest shift toward b12 resistance. However, while the K373R/D386N mutant conferred optimal b12 resistance, the triple mutant that carried T388V to abrogate N386 as a PGS was sensitive to b12. These observations confirm that the glycan at N386 rather than the asparagine residue itself is responsible for b12 resistance. Intriguingly, optimal b12 resistance conferred by the N386 glycosylation site required the presence of an arginine at residue 373.

The mutants described above were all based on the B33 envelope and have implicated a PGS at residue 386 as important for b12 resistance. We next substituted T388 for alanine in b12-resistant LN40 env, leaving the N386 intact but eliminating the PGS. T388A rendered LN40 sensitive to b12, confirming the role of the N386 glycan as a critical determinant for LN40 resistance to b12 (Fig. 6C).

**Envelopes that carry the N386 glycosylation site may be sensitive or resistant to b12 depending on residue 373.** NA420 B42 envelope is from the same patient as are the B33 and LN40 envelopes. However, unlike B33, B42 carried a PGS at N386 and yet was sensitive to b12. Results presented above show that B33 required the presence of an arginine residue at 373 in addition to N386 for optimal resistance to b12. We therefore introduced an arginine at residue 373 in B42. M373R rendered B42 resistant to b12 (Table 1) and confirmed the importance of residue 373 for b12 resistance.

To further test the role of residue 373, we prepared LN40 mutants carrying M, T, or K (M and T are the most common residues) at residue 373 and B33 mutants carrying M, T, or R

TABLE 1. Role of envelope residue 373 in b12 neutralization

env	wt or mutant residues (373/386)	IC <sub>50</sub> <sup>a</sup> (μg/ml)
B42	MN (wt)	1
	RN	>50
B33	KD (wt)	1.7
	MN	1.7
	TN	2.5
	KN	6.1
	RN	>50
LN40	RN (wt)	>50
	MN	2.8
	TN	3.7
	KN	3.6

<sup>a</sup> IC<sub>50</sub>, 50% inhibitory concentration.

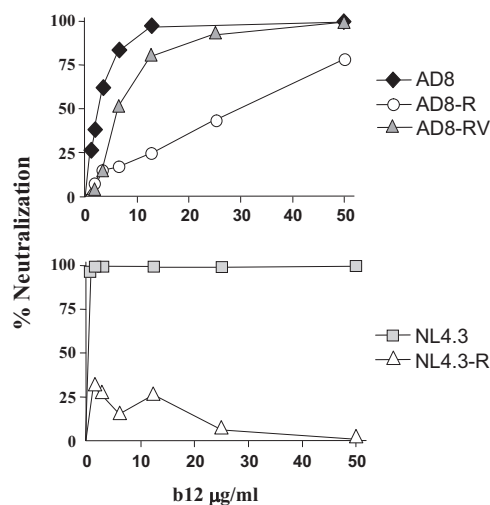


FIG. 7. R373 and the N386 glycan confer b12 resistance to NL4.3 and AD8. The introduction of an arginine at residue 373 conferred b12 resistance on both AD8 and NL4.3, both of which carry a PGS at N386. A valine was next introduced at residue 388 in R373<sup>+</sup> AD8 to eliminate the PGS at N386. This mutant was sensitive to b12 (top panel), showing that R373 and the N386 glycan act together to confer b12 resistance on NL4.3 and AD8.

(in conjunction with a PGS at N386). M and T represent the most prevalent residues at position 373. Only a combination of both R373 and N386 conferred optimal b12 resistance in both B33 and LN40 envelopes (Table 1). Combinations of M373 or T373 with N386 perhaps conferred a marginal shift in resistance compared to that conferred on B33, while K373/N386 conferred a modest shift toward resistance (Table 1).

We next introduced R373 into NL4.3 and macrophage-tropic AD8 envelopes, both of which are sensitive to b12 despite carrying PGSs at N386. AD8 usually has M at 373, while NL4.3 has T373. The presence of R373 rendered both NL4.3 and AD8 resistant to b12 (Fig. 7), indicating that variation at residue 373 can completely modulate b12 resistance for envelopes unrelated to B33 and LN40. Finally, we introduced a valine residue at position 388 into the AD8 envelope that already carried R373. This mutant thus carries R373 and N386; however, N386 is no longer a potential glycosylation signal. This AD8 mutant was sensitive to b12 (Fig. 7, top panel), confirming that R373 and the glycan at N386 act together in AD8 to confer b12 resistance.

**Residue 373 is adjacent to N386, and together these determinants may sterically block the formation of a b12:env complex.** The structure of the b12:gp120 complex was previously solved using gp120 from b12-sensitive HXBc2, which carries a threonine at 373 and a PGS at N386 (17). Our studies show that R373 was required with a PGS at N386 to protect B33 against b12, while K, T, or M at 373 failed to protect. Residues 373 and 386 are proximal in all the gp120 structures published to date including the unliganded simian immunodeficiency virus gp120 (1), gp120 complexed with CD4 and MAb 17b (7), and the gp120/b12 complex (17). Figure 8 shows the proximity of residues 373 and 386 in the gp120/b12 complex and reveals how the benzene ring of the W100 side chain on b12 targets a pocket surrounded by the side chain of residue 373 and the

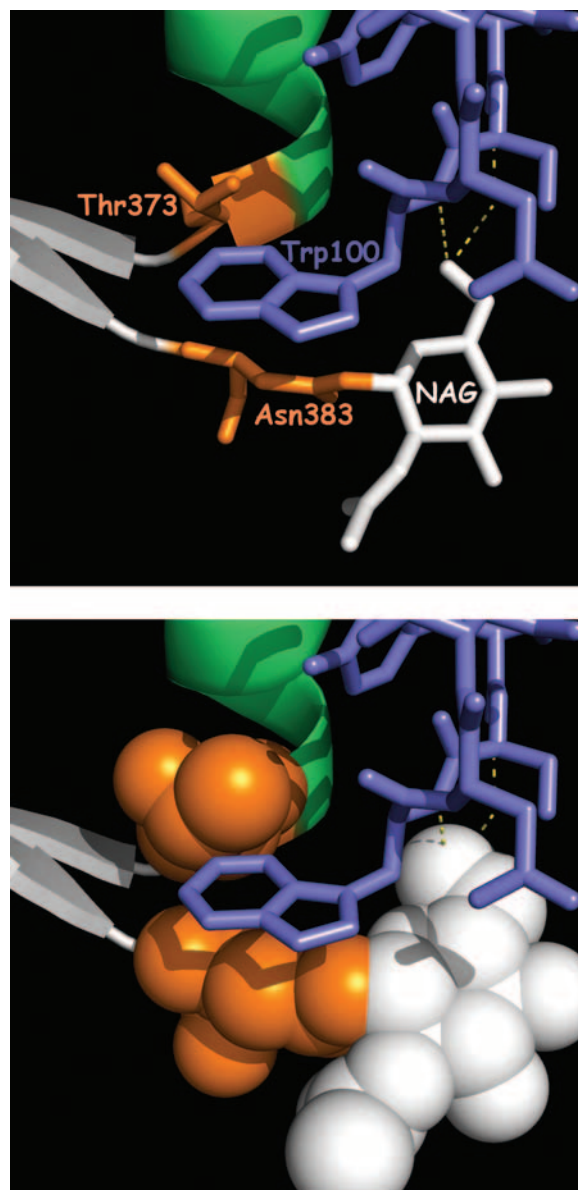


FIG. 8. Proximal gp120 residues R373 and N386 cooperate to sterically block b12 binding and neutralization. Residues 373 and 386 are proximal in the published structure of b12 complexed with gp120 as shown (17). In this reported structure, residue 373 is a threonine. The introduction of an arginine residue at this position using the PyMol program is not possible due to the steric limitations of residues surrounding this pocket. The presence of R373 and N386 is therefore likely to sterically exclude the benzene ring of b12 W100 from this pocket.

N386 glycan. Using the PyMol program, we tried to introduce an arginine at residue 373. However, arginine, which has a longer side chain than that of K, T, or M, does not fit into the b12:gp120 structure without nonpermissible interactions with surrounding residues (not shown). These observations strongly suggest that a combination of the glycan at N386 and the side chain of R373 sterically prevents an interaction with W100 on b12 with gp120 and the formation of a gp120/b12 complex (17).

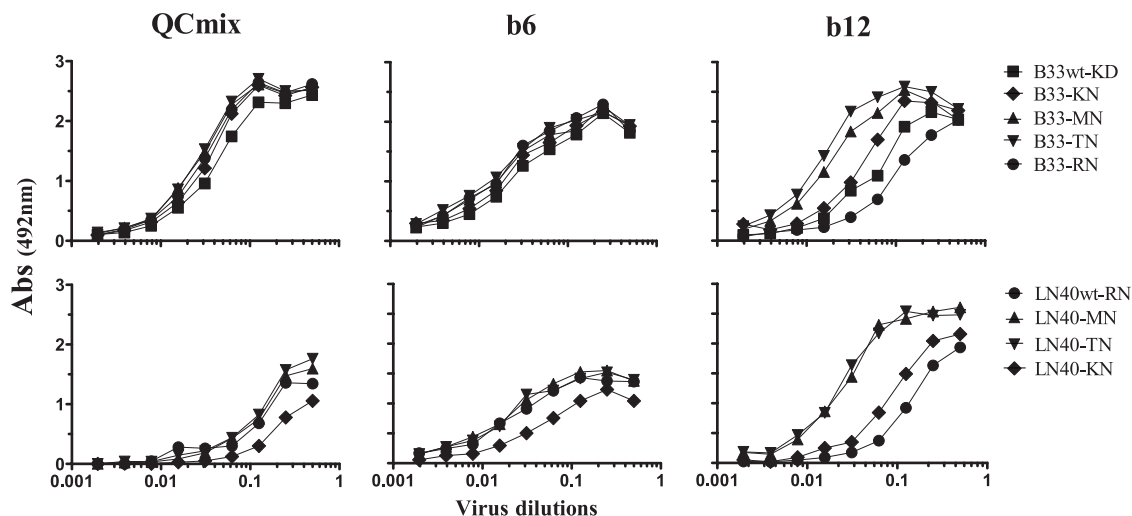


FIG. 9. b12 binding to captured, solubilized, monomeric gp120. Serial dilutions of env<sup>+</sup> pseudovirions were treated with Tween 20 and captured onto a solid phase as described in Materials and Methods. The capacities of b6, b12, and QCmix to bind captured B33 or LN gp120 were evaluated by ELISA. Generally b6 and QCmix bound to captured B33 or LN40 gp120s at equivalent levels. Binding of b12 to B33, LN40, and mutants that carried K, R, M, or T373 in combination with N386 varied. Generally, M and T373 (with N386) bound b12 better than did K or R373. Nevertheless, this modest variation in binding b12 does not explain the more complete modulation of neutralization sensitivity.

#### R373 and N386 reduce binding of b12 to monomeric gp120.

The structure of the b12:gp120 complex strongly suggests that the combination of R373 and the N386 glycan acts to prevent binding of b12. We next evaluated whether R373/N386 or combinations of these and other residues at these positions influence binding of b12 to monomeric, detergent-treated gp120. To test this, solubilized monomeric gp120, present in pseudovirions treated with 0.02% Tween 20, was captured onto 96-well ELISA plates using sheep Abs that recognize the C terminus of gp120. We tested whether b12, b6, or a mix of HIV-1<sup>+</sup> human sera (QCmix) could bind captured gp120. Both QCmix and b6 bound B33 wild type (wt) (K373/D386) and mutants K373/N386, M373/N386, T373/N386, and R373/N386 to similar levels (Fig. 9, top panels). b12 also bound to B33 wt and all mutants, although binding to R373/N386 was slightly less efficient than that for B33 wt gp120 and other B33 mutants. For LN40, the QCmix and b6 bound wt and mutants to similar levels except for the K373/N386 mutant, which was presumably present at lower concentrations in the pseudovirion preparation (Fig. 9, bottom panels). b12 also bound LN40 wt and each mutant, with slightly more efficient binding to the M373/N386 and T373/N386 mutants than to LN40 wt, which carried R373/N386. These observations show that R373 and N386 together reduce binding of b12 to monomeric gp120 solubilized in Tween 20. However, this modest reduction in b12 binding cannot explain the more complete resistance observed in neutralization assays. These observations suggest that 0.05% Tween 20 may allow b12 better access to the gp120 pocket flanked by residues 373 and 386 and/or that the trimeric form of functional envelope spikes contributes to the shielding of the b12 epitope by R373 and N386.

#### DISCUSSION

We have studied envelope determinants that confer natural resistance to b12, a CD4bs MAb. We investigated envelopes

previously amplified by PCR without culture from brain and LN tissue of the same patient (NA420). The brain-derived envelopes were sensitive to b12, while those from LN were resistant (12). We show that HIV-1 sensitivity to b12 can be completely modulated by the presence of a glycan at residue 386, although resistance required the presence of an arginine at residue 373. Together, R373 and the N386 glycan may sterically prevent the benzene ring of b12 W100 from penetrating a pocket proximal to these two residues. Nevertheless, b12 bound to monomeric, detergent-solubilized gp120 that carried R373/N386, indicating that the envelope trimer may also play a role in the protection of this epitope. The introduction of R373 into b12-sensitive envelopes NL4.3 and AD8 (which contain PGSSs at N386) rendered both resistant to b12, confirming that this mechanism of b12 resistance transfers to unrelated envelopes. Our data extend the recent report by Dunfee et al., also using brain-derived envelopes, that showed a modest influence of N386 for b12 sensitivity (5). Nonetheless, it is clear that many HIV-1 envelopes (either b12 resistant or sensitive) carry a PGS at N386, indicating that the presence of N386 alone does not predict b12 sensitivity. Interestingly, in the crystal structure of the gp120:b12 complex (17), the glycan at N386 contacts b12, even though we show here that envelopes lacking N386 are sensitive to b12. Thus, the glycan at N386 is not required for b12 binding or neutralization. On the HIV sequence database (<http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html>) methionine and threonine (which do not confer b12 resistance) are the most common residues at position 373. Arginine and lysine (residues at 373 for LN40 and B33, respectively) have larger side chains than methionine and threonine. These residues are rare in the database, suggesting that they may adversely affect viral fitness. K373 in combination with N386 conferred partial resistance on B33 (Table 1). However, the larger side chain of R373 together with the N386 glycan may fill the proximal pocket and generally confer b12



resistance, as shown for AD8, NL4.3, B33, B42, and LN40 envelopes.

Our data also show a partial but significant shift in b12 sensitivity that is conferred by residues on the N-terminal flank of the CD4 binding loop. This region is variable and may play a role in the exposure of the CD4 binding loop, perhaps by altering its orientation. Interestingly, residues in the V3 loop also contributed to this partial resistance to b12. It is therefore possible that the V3 loop in the unliganded envelope lies close enough to the CD4 binding loop to influence its exposure, although other mechanisms are also tenable. Recently, Lynch et al. reported that a single change in the V3 loop of a clade C envelope conferred exposure of the CD4 binding site and sensitivity to soluble CD4 (8). These observations also suggest a role for the V3 loop in protecting the CD4 binding site. Using the same B33 and LN40 envelopes described here, we have recently investigated envelope determinants that confer R5 macrophage tropism. We showed that determinants affecting R5 macrophage tropism essentially overlap with those reported here for b12 sensitivity. However, R373 and N386 have only a modest influence on R5 macrophage tropism, while the determinants on the N-terminal flank of the CD4 binding loop are more critical (M. J. Duenas-Decamp et al., unpublished observations). Nevertheless, together these results strongly support a hypothesis where the evolution of envelopes in the brain that confer enhanced macrophage infection directly results in increased sensitivity to neutralizing Abs that target the CD4bs. Conversely, replication in immune tissues such as LNs selects for variants where the CD4bs is protected from CD4bs Abs such as b12. The variation in b12 sensitivity reported here and in a more extensive study (12) thus supports the possibility that Abs recognizing the CD4bs or proximal epitopes are present *in vivo* and are an important selective force that impacts on R5 macrophage tropism and neutralization sensitivity.

In summary, we describe important mechanisms exploited by HIV-1 to protect the CD4 binding site from neutralizing Abs. We have identified determinants on the N-terminal flank of the CD4 binding loop that partially modulate b12 sensitivity and likely influence the exposure or orientation of this loop. In addition, we show that a combination of R373 and a glycosylation site at N386 acts to sterically block W100 of b12 penetrating a pocket proximal to these two residues. N-linked glycans have been reported to protect against neutralizing Abs. However, our data show that additional envelope determinants modulate the capacity of glycans to provide such protection. Our results greatly improve our understanding of envelope sensitivity to b12 neutralization and may lead to improved ways to design optimal CD4 binding site structures for vaccines.

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